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Insulin-like growth factor type-1 receptor down-regulation associated with dwarfism in Holstein calves

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Abstract

Perturbations in endocrine functions can impact normal growth. Endocrine traits were studied in three dwarf calves exhibiting retarded but proportionate growth and four phenotypically normal half-siblings, sired by the same bull, and four unrelated control calves. Plasma 3,5,3′-triiodothyronine and thyroxine concentrations in dwarfs and half-siblings were in the physiological range and responded normally to injected thyroid-releasing hormone. Plasma glucagon concentrations were different (dwarfs, controls > half-siblings; $P < 0.05$). Plasma growth hormone (GH), insulin-like growth factor-1 (IGF-1) and insulin concentrations in the three groups during an 8-h period were similar, but integrated GH concentrations (areas under concentration curves) were different (dwarfs > controls, $P < 0.02$; half-siblings > controls, $P = 0.08$). Responses of GH to xylazine and to a GH-releasing-factor analogue were similar in dwarfs and half-siblings. Relative gene expression of IGF-1, IGF-2, GH receptor (GHR), insulin receptor, IGF-1 type-1 and -2 receptors (IGF-1R, IGF-2R), and IGF

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binding proteins were measured in liver and anconeus muscle. GHR mRNA levels were different in liver (dwarfs < controls, $P < 0.002$; dwarfs < half-siblings, $P = 0.06$; half-siblings < controls, $P = 0.08$) but not in muscle. IGF-1R mRNA abundance in liver in half-siblings and controls was 2.4- and 2.5-fold higher ($P = 0.003$ and $P = 0.001$, respectively) and in muscle tissue was 2.3- and 1.8-fold higher ($P = 0.01$ and $P = 0.08$, respectively) than in dwarfs. Hepatic IGF-1R protein levels (Western blots) in muscle were 2.5-fold higher ($P < 0.05$) and in liver and muscle (quantitative immunohistochemistry) were higher ($P < 0.02$ and $P < 0.07$, respectively) in half-siblings than in dwarfs. The reduced presence of IGF-1R may have been the underlying cause of dwarfism in studied calves.

1. Introduction

Fundamental peptide elements involved in growth regulation include members of the somatotropic axis (SA), consisting of growth hormone releasing factor (GRF), somatostatin, growth hormone (GH), insulin-like growth factors-1 and -2 (IGF-1 and IGF-2), and IGF-binding proteins (IGFBPs)-1 to -6. In addition, specific receptors to the peptide elements mediate activity and include the growth hormone receptor (GHR) and the IGF type-1 and -2 receptors (IGF-1R and IGF-2R) of which IGF-1R shares structural homology with the insulin receptor (IR). The GH secreted from the pituitary gland influences the production of hepatic (and in part extra-hepatic) IGF-1 in large part through GHR. IGFs exert their effects in skeletal muscle, liver, bone, fat tissue and gastrointestinal tract through IGF-1R, IGF-2R or IR [1]. While other factors [including insulin, glucagon, 3,5,3′-triiodthyronine (T_3), thyroxine (T_4), and glucocorticoids] also mediate growth processes through their own receptor-based actions, they also have a gating function within the SA affecting in many instances the functional responsiveness of cells to GH in the regulation of IGF-1 and metabolism.

Genetic defects within elements of the SA may cause either enhanced or reduced growth. There may be reduced secretion or also a defect of the GH gene or peptide [2] as a cause for dwarfism. The historically classical documented growth perturbation was the GHR defect in humans characterized by high plasma concentrations of GH, but low plasma concentrations of IGF-1 [3,4] and defects or complete lack of the IGF-1R are thought to be a causative factor in retarded human growth [5–8].

Dwarfism has also been well documented in several domestic animal species (dogs, pigs, cattle, poultry) and has been in part shown to be associated with perturbations in the SA [9–12]. The phenotype in the bovine may be manifested as either disproportional or much less frequently as proportional growth retardation [12–29]. Few studies have been reported which identify the causative genetic mutations for the observed growth retardation in cattle, but in some an involvement of the SA axis has been suggested or documented [21,23,25,26,28,29]. Receptor defects in association with growth retardation have also been described, such as within the GHR gene in a line of miniature *Bos indicus* [28]. A decreased amount of hepatic mRNA for GHR was identified and indicated to be due to a promoter defect of the GHR gene. Small cattle breeds are, however, not necessarily characterized

by defects of the SA axis. Thus, Dahomey cattle are normally a small and proportionally growing breed that reaches a final body weight of only 150–200 kg, but plasma GH and IGF-1 concentrations were in the normal range of Holstein-Friesian, Red-Holstein × Simmental and Brown Swiss cattle (Blum, unpublished observations).

The objective of the present investigation was to profile blood plasma metabolic and endocrine traits, mRNA expression of members of the SA, and levels of IGF-1R in tissues (liver, muscle) obtained from dwarf progeny of a single red and white German Holstein bull, in order to determine whether changes in plasma concentrations, gene expression of members of the SA or levels of IGF-1R in liver and muscle are associated with and may be the primary cause of this congenital condition in cattle.

2. Materials and methods

2.1. Animals

Experimental procedures were approved by the research advisory committee of the government of Hannover, Germany, and followed the guidelines and laws on animal protection. Three red and white German Holstein calves, born between 10 May 2003 and 01 October 2003 at a farm in Schleswig-Holstein, Germany, were identified as being congenital dwarfs (two males and one female) and exhibited retarded, but proportional growth. All three calves were sired by a single natural service bull, which was born on 21 October 2000 and slaughtered at 09 March 2003 (Fig. 1). One of these dwarfs (Nepumuk, a male) died on 16
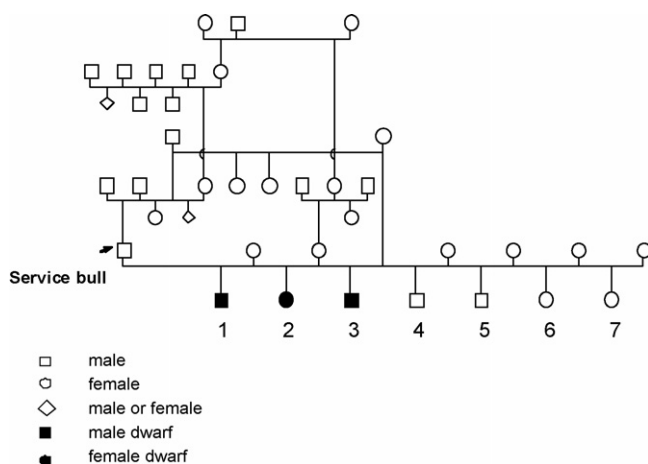

Fig. 1. Pedigree chart for dwarf calves and their half-siblings. At the bottom close symbols represent the three dwarfs and open symbols the four half-siblings. Numbers on figure, names, ear tag numbers, sex, and birth dates of individual calves: 1 = Poldi (53528; male; born 10 May 2003); 2 = Lulu (53529; female; 29 June 2003); 3 = Nepumuk (53532; male; born 01 October 2003); 4 = Björn (53531; male; born 18 October 2003); 5 = Benni (53533; male; born 25 October 2003); 6 = Anna (53543; female; born 04 November 2003); 7 = Anita (53538; female; born 27 November 2003).

September 2005 and the two (normal male) half-siblings were slaughtered on 30 November 2005. In none of these calves were any abnormalities found. Four additional paternal half-siblings (two males, two females) with apparently normal growth patterns and born between 18 October 2003 and 27 November 2003 were also studied, as well as four female unrelated black and white German Holstein calves (controls), born between 09 May 2003 and 17 November 2003. Mitochondrial DNA analyses of available family members revealed no maternal relationships between the three dwarf calves. According to the farmer's record in addition eight calves sired by this bull with apparently normal growth were born between 17 July 2003 and 06 December 2003 and 16 other calves without phenotype records were either premature (by about 3 weeks) or stillborn.

2.2. Housing, feeding and health status

The three dwarfs and four half-siblings were moved on 15 January 2004 from the private farm to the Institute for Animal Breeding and Genetics of the University of Veterinary Medicine Hannover. There they were kept on straw without slatted floor. Until the age of 3.5 months they were fed a milk replacer (Sprayfo Blau, Diepholz, Germany) and starting at the age of 4 weeks they were also fed hay *ad libitum*. Subsequently calves were fed grass silage with hay of average quality *ad libitum* and were offered concentrates [0.5 kg/(animal × day)], which contained (per kg dry matter) 6.7 MJ net energy, 160 g crude protein, 120 g crude fiber, 35 g crude fat, and 120 g ash.

2.3. Sample collection

2.3.1. Blood samples

Up to four samples were taken from a jugular vein between mid February and mid April 2004 to measure clinico-chemical traits. Samples (10 mL) were either anticoagulated for hematological analyses or for plasma collection or contained no anticoagulants for serum collection.

Blood samples (5 mL), anticoagulated with EDTA (1.8 mg/mL blood), for GH, insulin, glucagon, and IGF-1 analyses were collected in March 2004 through a jugular vein catheter. The samples for the determination of GH and insulin were drawn every 20 min from 08:00–16:00; samples for measurements of glucagon and IGF-1 were drawn at 08:00 and 16:00.

After the 8-h sample collection period, central α_2-adrenoceptor stimulation testing of GH release [30] was additionally performed using xylazine (23.3 mg as xylazine chloride/mL or 20 mg xylazine/mL; Burgdorf, Germany). Xylazine was intravenously injected (200 μg/kg body weight) at time 16:00, followed by additional blood sampling every 15 min for 1 h for GH and insulin determinations. Glucagon and IGF-1 concentrations were determined before treatment and at 1 h post-treatment. Plasma samples were stored at −20 °C until analyzed.

Dynamic pituitary challenge testing for GH secretory response was performed in August 2005. Blood samples (2 mL; anticoagulated with 1.8 mg EDTA/mL blood) were obtained through a jugular vein catheter from both dwarfs and half-siblings at 10 and 0.5 min before and at 5, 10, 20, 30, 45, 60, 90, and 120 min after the intravenous injection of a GH releas-

ing factor analog [Ile^2-Ser^8-Ala^{15}-Ser^{28}-Hse^{30} bovine GRF-(1-30)-NH-ethyl acetate salt; 0.2 μg/kg body weight; kindly donated by W.M. Moseley, Kalamazoo, MI]. Plasma samples were stored at −20 °C until analyzed.

Further dynamic testing of the thyroid axis in response to intravenous administration of thyrotropin releasing hormone (TRH) was performed in November 2004. After collecting the first blood sample, 2 mL of TRH (Protirelin, 0.2 mg/mL; A.G. Ferring, Kiel, Germany), blood samples (2 mL; anticoagluated with 1.8 mg EDTA/mL blood) were drawn at 3, 4, 5 and 6 h after the administration of TRH through a jugular catheter for subsequent determinations of T_3 and T_4 concentration changes. Plasma samples were stored at −20 °C until analyzed.

2.3.2. Tissue samples

Samples of liver (2 mg × 60 mg tissue) were obtained in March 2004 and August 2005 by needle biopsy (diameter: 2.1 mm, length: 150 mm; Baxter Healthcare Cooperation, Valencia, CA) and of skeletal muscle (anconeus muscle; 6 mg × 60 mg tissue) by surgical interventions following local (subcutaneous) anesthesia. Samples obtained in March 2004 were placed immediately in Eppendorf microcentrifuge tubes filled with 1.5 mL RNA-later (Qiagen, Hilden, Germany), stored at 4 °C for 24 h and then at −20 °C for later mRNA analyses of members of the SA. Biopsy cores of liver (up to 8; 20–30 mg each) and muscle (up to 8; 20–30 mg each) obtained in August 2005 were frozen in liquid nitrogen and used for Western radioligand blot analyses of IGF-1R. In addition, respective samples of each tissue were stabilized and fixed in phosphate buffered saline containing paraformaldehyde (3–4%) for 12–14 h. Following incubation in ethanol (70%) for 24 h, tissues were imbedded into paraffin blocks for sectioning and later immunohistochemical analyses of IGF-1R.

2.4. Laboratory analyses

2.4.1. Blood analyses

Concentrations of GH, IGF-1 and insulin were measured by radioimmunoassay (RIA) as previously described [31–33]. For glucagon quantification an RIA kit was used (Linco Research Inc., St. Louis, MO), as described previously [33,34]. Plasma concentration of T_3 and T_4 were measured using an RIA kit (Diagnostic Products Corporation, Los Angeles, CA). Concentrations of hormones to be measured were mostly well within the middle range, i.e. in the most sensitive and precise regions of standard curve concentrations. Intra- and interassay coefficients of variation for all RIAs were below 10 and 15%, respectively.

The number of erythrocytes and leucocytes was determined with a coulter counter and the hematocrit with a microhematocrit centrifuge. Spectrophotometric methods were applied to determine concentrations or activities of total bilirubin and urea (with kits from Labor und Technik, Lehmann, Berlin), cholesterol, glucose, calcium, inorganic phosphorus, magnesium, alkaline phosphatase and aspartate-amino-transferase (with kits from MTI Diagnostics GmbH, Idstein, Germany), β-hydroxybutyrate (with a kit from Randox Laboratories, Crumlin, UK), γ-glutamyl-transferase and hemoglobin (with kits from Nihon-Kohden, Japan), and glutamate-dehydrogenase (with a kit from Roche Diagnostica, Mannheim, Germany). Inductively coupled plasma optical emission spectroscopy (Vista-Pro, Varian, Darmstadt, Germany) was applied to measure concentrations of sodium, potassium and

chloride (with kits from ABX Diagnostics, Montpellier, France), and iron, zinc and manganese (with kits from Merck, Darmstadt, Germany). The selenium status was evaluated indirectly through the determination of glutathione peroxidase activity. Concentrations of Vitamins A, E and B_1 were measured as described [35,36].

2.4.2. Karyotyping

Blood samples from dwarfs were prepared for metaphase spreads using phytohemagglutinin-stimulated blood lymphocytes. Cells were harvested and slides prepared using standard cytogenetic techniques. The chromosomes were GTG-banded and well-banded metaphase chromosomes were photographed using a highly sensitive CCD camera and IPLab 2.2.3 (Scanalytics Inc., Fairfax, VA). Identification adhered strictly to the ISCNDB 2000 classification [37].

2.4.3. Determination of tissue mRNA abundance

Total RNA was extracted from liver tissue with the RNA Isolation NucleoSpin® RNAII kit (Macherey-Nagel, Oensingen, Switzerland). The TRIzol™ method [38] was used for RNA extraction from muscle tissue. Two extractions were performed for each tissue at each time point. Amounts of the extracted RNA were determined by optical density (OD) measurements using a photometer (Eppendorf, Netheler-Hinz, Hamburg, Germany) at an OD of 260 nm. The purity of the RNA was verified by photometrical absorption (ratio of OD at 260/280 nm; ideal OD > 2, acceptable OD 1.7–2.1). Extracted RNA was stored at −80 °C until analyzed. One microgram of total RNA from the sample preparation was reverse transcribed using Promega Reverse Transcriptase with 10 mM random hexamer primers according to the instructions of the manufacturer (Promega Corporation, Madison, WI), as described previously [39].

The primers used for the generation of PCR products of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), GHR, IGF-1R, IGF-2R, IR, IGF-1, IGF-2, and IGFBP-1 to -6 were designed and validated previously [39,40] Table 1. The PCR quantification was performed with the LightCycler™ system using software package 3.3 (Roche Molecular Biochemicals, Rotkreuz, Switzerland) which utilizes SYBR Green I. Reaction components were prepared as follows: 6.4 μL H_2O; 1.2 μL $MgCl_2$ (4 mM); 0.2 μL forward primer (4 μM); 0.2 μL reverse primer (4 μM) and 1.0 μL LightCycler FastStart Mix containing FastStart Taq DNA polymerase, and DNA double-strand specific SYBR Green I dye for detection. One microgram cDNA (in 1 μL) was added as PCR template. The RT-PCR conditions for the amplification were: denaturation program (95 °C for 10 min), a four-segment amplification and quantification program repeated 36–50 times (denaturation at 95 °C for 15 s, annealing at 55 °C for 5 s, elongation and single fluorescence acquisition at 72 °C for 4 s per 100 bp), melting curve program (60–99 °C with a heating rate of 0.1 °C/s and continuous fluorescence measurements), and cooling down to 40 °C. For quantification of IGFBP-1, -2 and -3 cDNA in skeletal muscle tissue the RT-PCR conditions were modified as follows: for IGFBP-1, 35 cycles, annealing temperature 62 °C, elongation time 6 s; for IGFBP-2, as for IGFBP-1, but elongation time 5 s; for IGFBP-3, as for IGFBP-1, but 38 cycles, annealing temperature of 62 °C, and elongation time 8 s. If more than 35 cycles were needed to get a signal, the presence of the cDNA was considered to be too low and was defined as non-detectable. The RT-PCR of two independent cDNA extractions per tissue

Table 1
Forward (For) and reverse (Rev) primer sequences (5′ → 3′) and reverse-transcription polymerase chain reaction (RT-PCR) product length

Gene/mRNA	Primer sequence (5′ → 3′)	Length (bp)	References
GAPDH	For GTC TTC ACT ACC ATG GAG AAG G Rev TCA TGG ATG ACC TTG GCC AG	197	[41]
IGF-1	For TCG CAT CTC TTC TAT CTG GCC CTG T Rev GCA GTA CAT CTC CAG CCT CCT CAG A	240	[39]
IGF-2	For GAC CGC GGC TTC TAC TTC AG Rev AAG AAC TTG CCC ACG GGG TAT	205	[39]
IGF-1R	For TTA AAA TGG CCA GAA CCT GAG Rev ATT ATA ACC AAG CCT CCC AC	314	[39]
IGF-2R	For TAC AAC TTC CGG TGG TAC ACC A Rev CAT GGC ATA CCA GTT TCC TCC A	144	[39]
IR	For TCC TCA AGG AGC TGG AGG AGT Rev GCT GCT GTC ACA TTC CCC A	163	[39]
GHR	For CCA GTT TCC ATG GTT CTT AAT TAT Rev TTC CTT TAA TCT TTG GAA CTG G	138	[39]
IGFBP-1	For TCA AGA AGT GGA AGG AGC CCT Rev AAT CCA TTC TTG TTG CAG TTT	123	[39]
IGFBP-2	For CAC CGG CAC ATG GGC AA Rev GAA GGC GCA TGG TGG AGA T	136	[39]
IGFBP-3	For ACA GAC ACC CAG AAC TTC TCC TC Rev GCT TCC TGC CCT TGG A	194	[39]
IGFBP-4	For GCC CTG TGG GGT GTA CAC Rev TGC AGC TCA CTC TGG CAG	342	[40]
IGFBP-5	For TGC GAG CTG GTC AAG GAG Rev TCC TCT GCC ATC TCG GAG	257	[40]
IGFBP-6	For AGA AAG AGG ATT TGC CTT Rev TCC GGT AGA AGC CCC TAT	324	[40]

Abbreviations: GAPDH = glyceraldehyde-3-phosphate dehydrogenase; IGF = insulin-like growth factor; IGF-R = insulin-like growth factor receptor; IR = insulin receptor; GHR = growth hormone receptor; IGFBP = insulin-like growth factor binding protein.

for each gene and calf was performed in replicate. Each assay included two pooled samples as an internal positive control, two water controls, and two replicates containing mRNA as negative control. Efficiencies of PCR assays of the different traits ranged from 1.77 to 1.97 (mean 1.84; ideal 2.0).

The GAPDH was selected as a housekeeping gene or endogenous control in order to correct for loading differences, as done in previous studies [39,41] and because external standards were not used. Sequences and identities of the PCR products were previously determined [39–41] and sizes of the PCR products were confirmed by gel (1.8% agarose) electrophoresis.

2.4.4. Western ligand blotting for the determination of the IGF type-1 receptor

Relative amounts of IGF-1R in liver were determined by ligand (Western) blotting, using [^{125}I]-IGF-1 as a ligand as described previously [42]. Samples of liver and muscle were homogenized four times for 20 s with an Ultra-Turrax homogeneizer (T25, Janke & Kunkel, Staufen, Germany) on crushed ice, followed by centrifugation of the homogenate at $800 \times g$ for 10 min, of the supernatanat at $10,000 \times g$ for 10 min, and of the resulting supernatant at $100,000 \times g$ for 1 h. The remaining pellet was suspended by a motor-driven glass-teflon homogeneizer in ice-cold buffer (consisting of 50 mM Tris–HCl, 6 mM MgCl$_2$ and 1 mM EGTA, pH 7.4) and stored at $-80\,^{\circ}$C until assayed. Based on protein concentrations that were determined using a kit (BCA Protein Assay Reagent; Pierce, Rockford, IL), membrane suspensions were adjusted to final concentrations of 200 µg/mL in the same buffer as described above but that contained in addition 50 mM mannitol. Although in pretests IGF-1R could be detected if only 25–50 µg of protein from livers were loaded on gels, it was decided to load gels with 200 µg protein/sample for safe detection. Three independent analyses were performed in which materials (liver, muscle) from always one dwarf and one randomly selected half-sibling were analyzed in the same run. The assays were made together with protein molecular weight markers and in the absence and presence of excess unlabeled IGF-1. The labeled band completely disappeared if excess non-radioactive IGF-1 (1 mg/mL) was added. Densities of bands were evaluated using a Bio Rad 6S-710 Imaging Densitometer after ligand blots were scanned with a Multiscan W900 (Sony). Four independent ligand blots, always with one randomly selected dwarf and one randomly selected half-sibling were done, but only three of them could be used; because of technical problems analyses were not possible in the liver of one of the female half-siblings. It was also not possible to detect IGF-1R using Western blotting in biopsies of muscles.

2.4.5. Immunohistochemical analyses for the IGF type-1 receptor

To demonstrate the IGF-1R by immunohistochemistry, a rabbit anti-IGF-1R antibody was used that was directed to the extracellular membrane-binding domain of the β-chain (Santa Cruz Biotechnology, Santa Cruz, CA) at 2.0 µg/mL. Standard protocols were used for dewaxing paraffin-embedded muscle and liver tissues, rehydration to aqueous buffer, and elimination of endogenous peroxidase/pseudoperoxidase activity, slide transfer to Tris-buffered saline containing 0.05% Triton-X-100, followed by baths in Tris–saline. Non-specific binding was blocked using 5% normal goat serum in Tris–saline, followed by incubation overnight with the primary antibodies. The IGF-1R antigen was visualized using the ABC method (Vectastain Elete® Rabbit Antibody Kit, Vector Laboratories, Burlingame, CA) with DAB as the substrate. Nuclei were counterstained for 2 min using Curazzi's hematoxylin. Quantitative image analysis was performed as previously validated and published [43]. Three fields for each of two to four specimens per slide for each animal were captured using an Olympus BX-40 microscope fitted with an Olympus DP-70 digital camera (for liver 20× objective, 200× final electronic magnification; for muscle 100× objective, 1000× final electronic magnification). In order to ensure uniformity of the digital analysis of each slide, all images for a given tissue and for each animal were composed into a master image using Adobe Photoshop® CS (Adobe Systems Inc., San Jose, CA), and that master image was subjected to a global enhancement of brightness and contrast necessary

to achieve valid color-specific (DAB-reddish brown) pixel identification and quantification. The individual frames of the composite image were then analyzed using the Image-Pro Plus® Image Analysis Software (Version 4.5.1, MediaCybernetics Inc. Silver Spring, MD) through a standardized protocol previously validated and published [43]. The intensity and pixel count of DAB stained cells was obtained by defining a color-cube-based segmentation option using the following rubric. Segmentation criteria defined the analysis in terms of $>3 \times 3$ pixel acceptance to eliminate background and non-specific DAB trace signal, sensitivity = 4, pixel color = red; measurements were the summated density of a field, with four-connect resolution and a single pass of a sharpening filter at 3×3 pixels. Each estimate of tissue antigen staining intensity for an individual animal was obtained as the average of the summated pixels/field of the images captured for each different tissue or biopsy specimens per slide.

2.5. Statistical analyses

Calf was the experimental unit for statistical analyses. Normal distribution of data was determined using the univariate procedure of SAS [44]. Log transformations were used to normalize data for IGFBP-4 in liver and muscle and for GHR and IGF-2 in muscle only. Group (dwarfs, half-siblings, controls) was the fixed effect. The model included group effects on mRNA abundance of SA genes and on clinico-hematological traits. The general linear model of SAS (GLM) was used to calculate ANOVA. When the ANOVA was statistically significant ($P < 0.05$), means were separated using the Bonferroni test. Means of four separate analyses of mRNA for each animal were utilized for the statistical analysis. Means of triplicate analyses (of GH, IGF-1, insulin, T_4, T_3) or duplicate analyses (of glucagon) were the basis for statistical analyses of endocrine traits. For analysis of pulsatile secretion of GH a pulse analysis program (PULSAR; [45]) was used. The areas under the curve (AUC) of GH values of 8-h profiles and the 2-h profiles after the administration of GRF-(1-30)-NH-ethyl acetate were calculated with the GraphPad computer program (GraphPad, San Diego, CA). Group differences for GH concentrations (after the administration of GRF-(1-30)-NH-ethyl acetate) and for T_3 and T_4 concentrations (after the administration of TRH) over time were evaluated using the SAS repeated measures procedure (time series analysis). Statistical analysis of the immunohistochemical data was performed after a $\log_{10}$ transformation of the data to eliminate a heterogeneity of variance inherent in the magnitude of the means between dwarfs and their half-siblings. The general linear models procedure of SAS was used and values represented as least squares means. Age was not included in the model.

3. Results

3.1. Growth profiles, body conformation traits, hematological and metabolic (clinico-chemical) traits, and health status

Growth profiles (up to early 2004) of the three dwarf calves showed marked differences compared with half-siblings and unrelated controls. Body weights of dwarfs were clearly
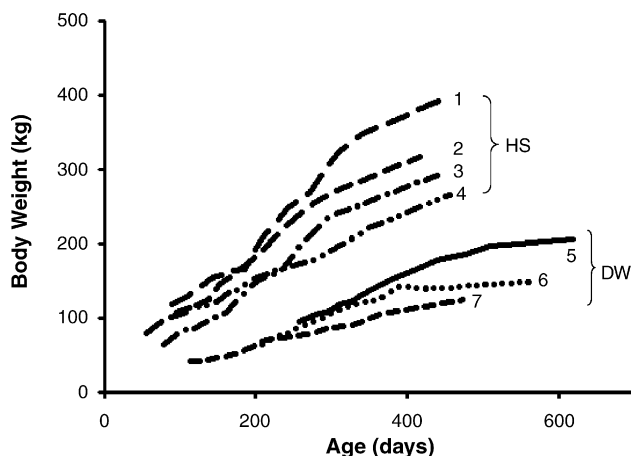
Fig. 2. Body weight of three dwarf (DW) and four half-sibling (HS) calves dependent on age. Numbers on figure: 1 = Benni; 2 = Björn; 3 = Anita; 4 = Anna; 5 = Poldi; 6 = Lulu; 7 = Nepumuk. Mean body weights on 13 April 2004 of dwarfs were 98 kg (Poldi 131 kg; Lulu 101 kg; Nepumuk 62 kg) and of half-siblings were 134 kg (Benni 165 kg; Björn 139 kg; Anna 108 kg; Anita 125 kg). Mean body weights in August 2005 of dwarfs were 181 kg (Poldi 202 kg; Lulu 193 kg; Nepumuk 147 kg) and of half-siblings were 456 kg (Benni 606 kg; Björn 415 kg; Anna 386 kg; Anita 417 kg). Control animals could be weighed only occasionally and are therefore not shown.

reduced compared with half-siblings (Fig. 2 and Table 2). At the age of 10 months, body weights in dwarfs, half-siblings and controls were 101 ± 9, 245 ± 12, and 277 ± 15 kg, respectively, and up to the age of 10 months the average daily gains of dwarfs, half-siblings and controls were 0.317 ± 0.029, 0.703 ± 0.211, and 0.830 ± 0.081 kg/day, respectively. Mean body weights reached in August 2005 of dwarf calves were much lower ($P < 0.001$) than of half-siblings (181 and 456 kg, respectively). Despite retarded growth, at a similar age (265 ± 10 days), dwarfs showed the same body proportions compared with half-siblings and control calves (Table 2), i.e. the ratios of hip height/metacarpus length as well as withers height/hip (*trochanter major*) breadth were not different among calves ($P > 0.5$).

Computer tomography and magnetic resonance imaging of the head region revealed no morphological defects (A. Jaggy, Division of Clinical Neurology, Department of Clinical Veterinary Medicine, School of Veterinary Medicine, University of Bern; personal communication). Animals at the time of blood and tissue sampling were healthy and no signs of clinical disease could be observed. The male dwarf calf that died did not exhibit gross or

Table 2
Body proportions at similar ages (265 ± 10 days) for dwarf (DW), half-sibling (HS) and a control calvf (C)

Group	Age (days)	Body weight (kg)	Hip height/metacarpus length	Withers height/hip breadth
DW ($n = 3$)	262 ± 5	$88 b \pm 5$	1.7 ± 0.03	3.0 ± 0.05
HS ($n = 3$)	269 ± 8	$242 a \pm 21$	1.7 ± 0.03	3.0 ± 0.1
CO ($n = 1$)	263	$254 a$	1.6	3.1

Data are means $\pm$ S.E.M. Different letters indicate significant differences ($P < 0.05$) among groups.

histopathological signs of a specific disease and there was no evidence for an association with the reduced growth rate.

As shown in Table 3, based on up to four samples per animal dwarf calves compared with half-siblings and control calves displayed slightly higher ($P < 0.05$) concentrations for hemoglobin, hematocrit, total bilirubin, and activities of aspartate-amino-transferase and glutamate-dehydrogenase. Additionally, plasma sodium concentrations were higher ($P < 0.05$) in dwarfs than in half-siblings.

3.2. Karyotyping

Analysis of GTG-banded chromosomes revealed a normal female chromosomal constitution of $2n = 60$, XX in the dwarf female and $2n = 60$, XY in two dwarf male calves, respectively. Using light microscopy no structural abnormalities like deletions or translocations were seen in the investigated metaphase spreads.

3.3. Plasma concentrations of GH, IGF-1, insulin, glucagon, T_4 and T_3

Across the 8-h sampling period, mean GH concentrations tended to be higher ($P = 0.09$) in dwarfs than in controls (Fig. 3 and Table 4). However, mean GH concentrations of dwarfs were not different from those of half-siblings ($P = 0.29$) and there were no statistically significant differences ($P > 0.10$) among dwarfs, half-siblings and controls for basal GH, number of GH peaks, and GH peak heights during the 8-h sampling period. Calves of all three groups responded similarly to xylazine with an increase ($P = 0.0008$) of plasma GH concentrations. Mean plasma GH concentrations for the three groups were not significantly different ($P > 0.05$). Of the three dwarf calves, one exhibited a reduced GH response compared with the other two dwarfs after the administration of xylazine. The GH profile of that dwarf during the 8-h sampling period (calf no. 3; Fig. 3) revealed a GH peak prior to the onset of the xylazine treatment, which may have negatively affected that calf's response to xylazine. Therefore, in order to obtain a better estimate of the total amount of GH produced by these calves, the area under the concentration curve (AUC) for both the 8-h sample and the subsequent xylazine stimulation test were calculated together. This combined analysis revealed that total AUC for GH was significantly higher ($P = 0.02$) in dwarfs and tended to be higher ($P = 0.08$) in half-siblings than in control calves, but AUC was not different between dwarfs and half-siblings (Table 4).

Concentrations of GH before the administration of GRF-(1-30)-NH-ethyl acetate were similar in dwarfs and half-siblings (Fig. 4). After the administration of GRF-(1-30)-NH-ethyl acetate, concentrations of GH in dwarfs increased above pre-injection levels ($P < 0.05$) and remained elevated for 30 min. A similar trend ($P < 0.1$) toward increased GH was observed in half-siblings. Concentrations at 30 and 45 min and AUC (from 0 to 120 min) were numerically, but not significantly higher ($P < 0.135$) in dwarfs than in half-siblings.

Concentrations of IGF-1 (data not shown) during the 8-h period remained stable and in dwarfs (229 ± 80 ng/mL), half-siblings (140 ± 23 ng/mL) and controls (293 ± 35 ng/mL) were not different ($P > 0.05$). Following the xylazine treatment (sample taken at 1 h after treatment), IGF-1 concentrations were not different among groups ($P = 0.86$).

Table 3
Blood hematology and clinical chemistry for three dwarfs (DW), four half-siblings (HS) and four controls (CO)

Traits	Reference values	Dwarfs ($n=3$)	Half-siblings ($n=4$)	Controls ($n=4$)
Leukocytes ($\times10^6$/mL)	4–12[a]	9.22 (5.7–14.0)	10.66 (6.50–13.70)	90.25 (6.90–11.80)
Erythrocytes ($\times10^9$/mL)	5.0–10.0[a]	10.4 (8.5–12.6)	10.0 (8.4–11.9)	9.4 (8.1–10.2)
Hemoglobin (g/L)	9.0–14.0[a]	121 (100–139) a	102 (91–115) b	106 (93–114) ab
Hematocrit (L/L)	280–380[a]	341 (231–391) a	283 (261–327) b	291 (251–314) ab
Total bilirubin (μmol/L)	<6.8[a]	7.7 (3.2–9.8) a	6.1 (3.4–8.3) ab	5.0 (2.5–6.2) b
Aspartate-amino-transferase (U/L)	<80[a]	44.3 (30.0–65.0) a	26.9 (22.0–34.0) b	29.5 (22.0–38.0) b
Gamma-glutamyl-transferase (U/L)	<27[a]	10.8 (5.0–17.0) a	12.4 (5.5–19.0) b	10.9 (5.0–15.0) b
Glutamate-dehydrogenase (U/L)	<10[a]	22.6 (8.2–56.9)	8.3 (4.5–15.7)	7.5 (3.4–18.0)
Alkaline phosphatase (U/L)	<315[a]	385.5 (212.0–732.0)	271.2 (141.0–512.0)	n.d.
Cholesterol (mmol/L)	1.3–3.9[a]	2.9 (1.6–4.0)	2.8 (1.3–4.4)	3.3 (2.0–2.9)
Glucose (mmol/L)	2.5–3.3[a]	5.3 (3.5–7.0)	4.7 (4.0–5.2)	4.2 (5.0–5.8)
β-Hydroxybutyrate (mmol/L)	<1.0[b]	0.43 (0.22–0.66)	0.57 (0.34–0.94)	0.45 (0.29–0.57)
Urea (mmol/L)	3.3–5.0[a]	5.0 (2.5–7.1)	3.9 (2.4–6.5)	3.9 (3.1–4.4)
Calcium (mmol/L)	2.2–2.9[a]	2.7 (2.4–3.0)	2.8 (2.6–2.9)	n.d.
Magnesium (mmol/L)	0.8–1.2[a]	0.85 (0.70–0.94)	0.86 (0.76–0.96)	n.d.
Inorganic Phosphorus (mmol/L)	2.0–3.5[a]	2.5 (2.3–2.8)	2.3 (1.9–2.7)	n.d.
Sodium (mmol/L)	135–157[a]	145.0 (138.0–152.0) a	139.8 (135.0–144.0) b	n.d.
Chloride (mmol/L)	90–110[a]	95.7 (90.0–104.0)	99.1 (97.0–101.5)	n.d.
Iron (μmol/L)	25–40[b]	32.2 (20.1–46.4)	27.0 (24.7–28.9)	n.d.
Zinc (μmol/L)	12.0–24.0[b]	15.4 (13.3–17.8)	14.0 (13.4–14.7)	n.d.
Selenium (μg/L)	>70.0[b]	95.8 (47.0–118.0)	96.5 (83.0–111.0)	n.d.
Manganese (μg/L)	>1.5[b]	2.8 (1.4–5.3)	2.4 (1.4–4.3)	n.d.
Vitamin A (mg/L)	>0.3[b]	0.7 (0.6–0.7)	0.6 (0.4–0.9)	n.d.
Vitamin E (mg/L)	>0.3[b]	4.9 (2.2–6.6)	5.4 (2.2–7.3)	n.d.
Vitamin B_1 (μg/L)	<40[b]	8.5 (5.8–13.8)	8.9 (6.1–10.4)	n.d.

Data of up to four samples per animal taken between mid February and mid April 2004, respectively. Data are means and ranges (shown in parentheses). n.d. = not done. Different letters indicate significant differences ($P<0.05$) among groups.
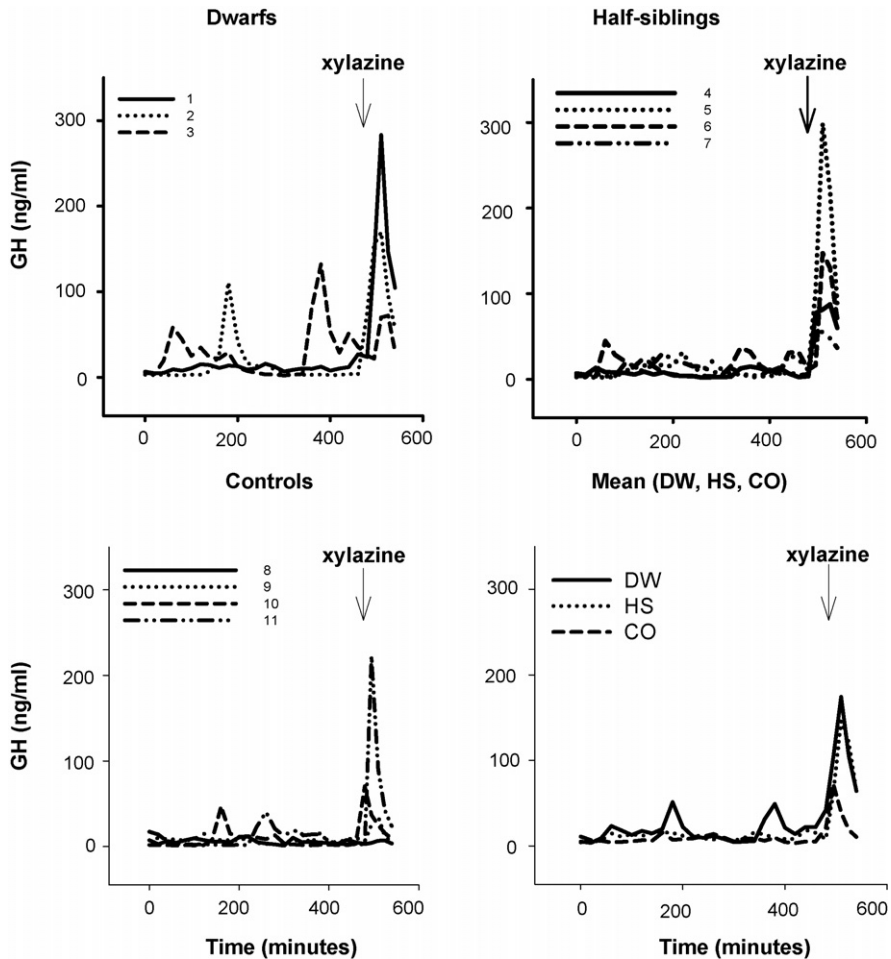
[a] From [46].
[b] From [47].

Fig. 3. Plasma concentration of growth hormone (GH) for three individual dwarfs (DW), four individual half-siblings (HS) and four individual controls (CO), and mean concentrations of the three DW, four HS and four CO collected from 08:00 to17:00. Between time 08:00 and 16:00 blood samples were taken every 20 min from a jugular vein. At time 16:00 xylazine (200 µg/kg body weight) was intravenously injected, followed by blood sampling every 15 min for 1 h. Numbers on figure: 1 = Nepumuk; 2 = Lulu; 3 = Poldi; 4 = Benni; 5 = Anna; 6 = Björn; 7 = Anita; 8 = Conni; 9 = Verena; 10 = Christine; 11 = Tina.

Insulin concentrations were similar ($P > 0.05$) in dwarfs, half-siblings and control calves (Fig. 5). The concentration of insulin in all groups decreased ($P = 0.0002$) after the administration of xylazine.

Glucagon concentrations (data not shown) remained stable over the 8-h period and in dwarfs (79 ± 13 pg/mL) and in controls (72 ± 6 pg/mL) were higher ($P = 0.02$ and $P = 0.04$, respectively) than in half-siblings (40 ± 4 pg/mL). Plasma glucagon concentrations similarly increased ($P < 0.002$) in all groups after xylazine injection.

Table 4
Plasma concentrations of growth hormone (GH) for three dwarfs (DW), four half-siblings (HS) and four controls (CO)

Parameters	DW	HS	CO
Mean (ng/mL)	18.2 ± 5.2	11.0 ± 1.7	8.0 ± 0.8
Number of peaks/8 h	2.0 ± 0	3.0 ± 0.4	2.8 ± 0.5
Peak height (ng/mL)	63.6 ± 25.9	18.9 ± 4.9	24.7 ± 11.7
Basal mean (ng/mL)	6.4 ± 1.8	4.8 ± 0.3	3.4 ± 0.8
AUC 8 h [ng/(min × mL)]	7536 ± 2467	4785 ± 657	3317 ± 370
AUC xylazine [ng/(min × mL)]	7874 ± 2134	6583 ± 1875	2528 ± 1188
AUC total [ng/(min × mL)]	15852 a ± 8010	11708 ab ± 2158	5947 b ± 1517

Data are means ± S.E.M. for samples taken during 8 h and for blood samples taken for 1 h after xylazine injections. Blood samples were taken between time 08:00 and 16:00 every 20 min followed by a xylazine injection and blood sampling every 15 min for 1 h. AUC 8 h: area under the concentration curve for 8 h (from 08:00 to 16:00); AUC xylazine: area under the concentration curve after the injection of xylazine at 16:00 (from 16:00 to 17:00); AUC total: area under the concentration curve for 9 h (from 08:00 to 17:00). Means with differences letters indicate significant differences ($P < 0.05$) among groups. AUC = area under the curve.

Concentrations of T_4 at time 0 were lower ($P = 0.009$) in dwarfs than in half-siblings and T_3 concentrations at time 0 ($P < 0.07$) tended also to be lower (Fig. 6) in dwarfs than in half-siblings. After the TRH treatment similar increments of T_3 and T_4 were observed in dwarfs and half-siblings, with dwarfs maintaining lower (though not statistically significant) concentrations during the sampling period. At 6 h after the TRH injection the concentrations of T_4 in the dwarfs were lower ($P = 0.04$) than in half-siblings. The T_3/T_4 ratios before as well as after the TRH administration were not different ($P > 0.05$) between dwarfs and half-siblings.
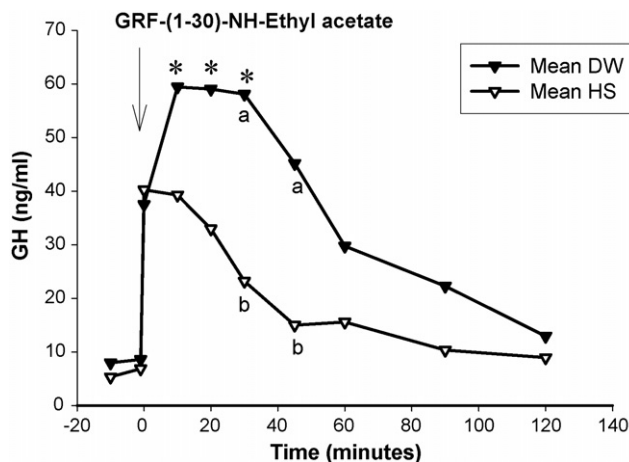

Fig. 4. Mean plasma concentrations of growth hormone (GH) for three dwarfs (DW) and four half-siblings (HS) after intravenous administration of GH-relasing factor analog GRF-(1-30)-NH-ethyl acetate (0.2 µg/kg body weight). (*) Indicates significant differences ($P < 0.05$) from basal (pre-injection) values in DW (there were no significant changes in HS). Different small letters indicate significant differences ($P < 0.05$) between DW and HS. Areas under the concentrations curves from 0 to 120 min were numerically higher, but not significantly ($P < 0.135$) in dwarfs than in half-siblings.
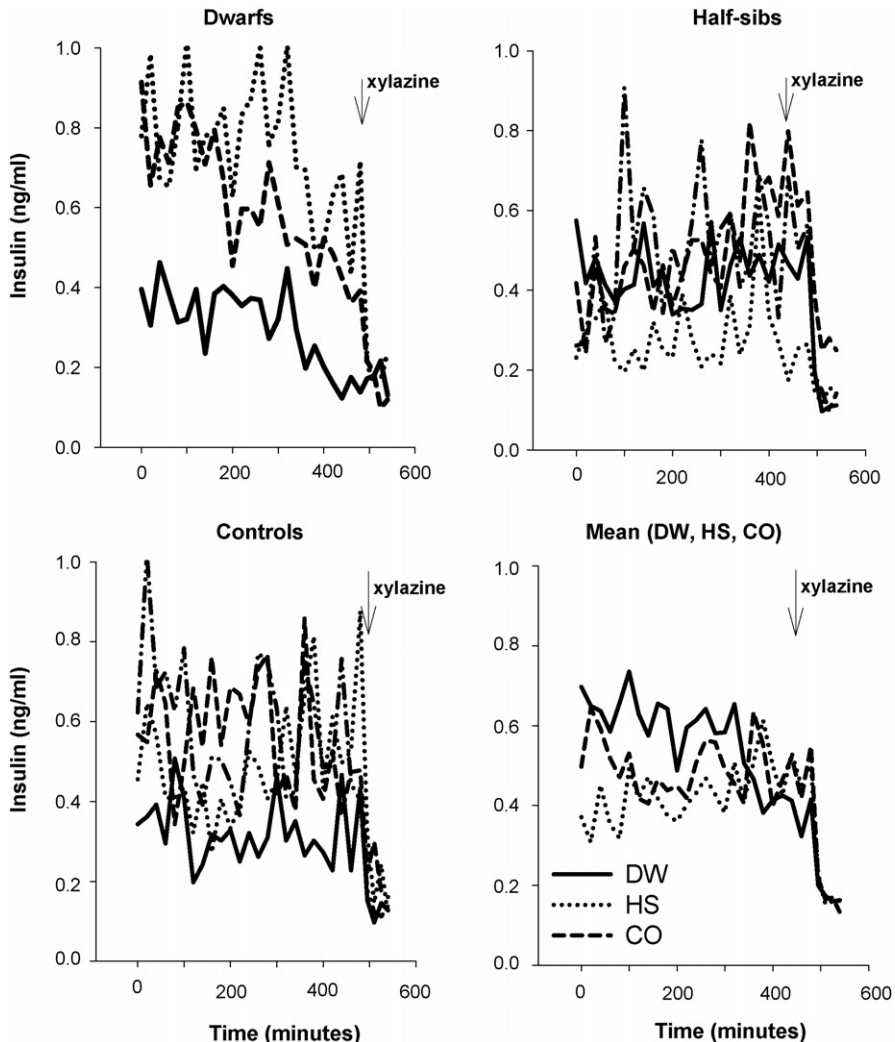
Fig. 5. Plasma concentration of insulin for three individual dwarfs (DW), four individual half-siblings (HS) and four individual controls (CO), and mean concentrations of the three DW, four HS and four CO from 08:00 to 17:00 collected from 08:00 to 17:00. Between time 08:00 and 16:00 blood samples were taken every 20 min. At time 16:00 xylazine was intravenously injected, followed by blood sampling every 15 min for 1 h. For identification of individual animals see Fig. 3.

3.4. Abundance of mRNA of GH receptor, insulin receptor, IGF-1 type-1 and -2 receptors, insulin-like growth factors-1 and -2, and IGF binding proteins in liver and skeletal muscle

Abundance of mRNA for several endocrine elements of the SA are presented in Table 5. GHR in liver tissue was lower ($P = 0.002$) in dwarfs than in controls, tended to be lower

Table 5

Relative mRNA abundance (values related to the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase) of growth hormone receptor (GHR), insulin receptor (IR), insulin-like growth factor-1 (IGF-1), IGF-2, IGF type-1 receptor (IGF-1R), IGF type-2 receptor (IGF-2R), and IGF binding proteins (IGFBP-1, -2, -3 and -4) using real-time reverse-transcription polymerase chain reaction (RT-PCR) for three dwarf calves (DW), four half-siblings (HS) and four controls (CO) in liver and skeletal (anconeus) muscle

Gene	Group					*P*-values		
	Tissue	Multiplication factor of data	DW (3)	HS (4)	CO (4)	DW vs. HS	DW vs. CO	HS vs. CO
GHR	Liver	$\times 10^{-6}$	7.1 b $\pm$ 0.4	9.8 ab $\pm$ 0.8	12.1 a $\pm$ 0.5	0.06	0.002	0.08
	Muscle	$\times 10^{-6}$	2.1 $\pm$ 0.2	2.2 $\pm$ 0.2	3.0 $\pm$ 0.5	1.0	0.4	0.3
IR	Liver	$\times 10^{-7}$	10.7 $\pm$ 1.3	10.6 $\pm$ 0.7	11.7 $\pm$ 0.9	1.0	1.0	1.0
	Muscle	$\times 10^{-7}$	5.3 $\pm$ 1.1	3.9 $\pm$ 0.3	6.9 $\pm$ 0.8	0.6	0.5	0.4
IGF-1	Liver	$\times 10^{-7}$	10.3 $\pm$ 2.8	16.8 $\pm$ 2.8	19.5 $\pm$ 3.1	1.0	0.2	0.8
	Muscle	$\times 10^{-7}$	0.7 $\pm$ 0.1	0.6 $\pm$ 0.06	0.6 $\pm$ 0.01	1.0	1.0	1.0
IGF-2	Liver	$\times 10^{-6}$	28.9 $\pm$ 1.7	30.4 $\pm$ 2.7	37.8 $\pm$ 1.6	1.0	0.07	0.11
	Muscle	$\times 10^{-6}$	7.4 $\pm$ 3.0	5.5 $\pm$ 0.7	5.1 $\pm$ 0.9	1.0	1.0	1.0
IGF-1R	Liver	$\times 10^{-8}$	7.4 b $\pm$ 0.3	21.7 a $\pm$ 2.3	24.5 a $\pm$ 2.1	0.003	0.001	1.0
	Muscle	$\times 10^{-8}$	12.5 b $\pm$ 1.3	25.6 a $\pm$ 4.0	23.2 ab $\pm$ 1.0	0.01	0.08	0.5
IGF-2R	Liver	$\times 10^{-9}$	6.9 $\pm$ 0.04	11.6 $\pm$ 2.0	8.6 $\pm$ 0.4	0.2	1.0	0.5
	Muscle	$\times 10^{-9}$	1.7 $\pm$ 0.3	1.0 $\pm$ 0.1	1.6 $\pm$ 0.2	0.12	1.0	0.14
IGFBP-1	Liver	$\times 10^{-7}$	349.1 $\pm$ 104.0	140.3 $\pm$ 46.9	349.4 $\pm$ 136.1	0.6	1.0	0.5
	Muscle	$\times 10^{-7}$	0.3 $\pm$ 0.1	0.6 $\pm$ 0.3	1.2 $\pm$ 1.0	1.0	1.0	1.0
IGFBP-2	Liver	$\times 10^{-7}$	168.4 $\pm$ 19.8	180.4 $\pm$ 14.9	111.7 $\pm$ 27.8	1.0	0.4	0.15
	Muscle	$\times 10^{-7}$	0.4 $\pm$ 0.3	0.6 $\pm$ 0.2	0.3 $\pm$ 0.2	1.0	1.0	0.7
IGFBP-3	Liver	$\times 10^{-8}$	68.4 $\pm$ 19.9	92.3 $\pm$ 10.9	57.2 $\pm$ 6.1	0.6	1.0	0.19
	Muscle	$\times 10^{-8}$	2.6 $\pm$ 0.3	4.4 $\pm$ 1.0	2.5 $\pm$ 0.3	0.26	1.0	0.17
IGFBP-4	Liver	$\times 10^{-7}$	0.9 $\pm$ 0.1	0.8 $\pm$ 0.10	1.13 $\pm$ 0.1	1.0	0.5	0.2
	Muscle	$\times 10^{-7}$	1.1 $\pm$ 0.1	1.0 $\pm$ 0.1	1.6 $\pm$ 0.2	1.0	1.0	0.4

Total RNA from each of the biopsies were extracted two times from both tissue liver and muscle. Data are means $\pm$ S.E.M. in percentage of glyceraldehyde 3-dehydrogenase (GAPDH) as housekeeping gene. Different letters indicate significant differences ($P < 0.05$) among groups. For abbreviations of the different genes see legend to Table 1.
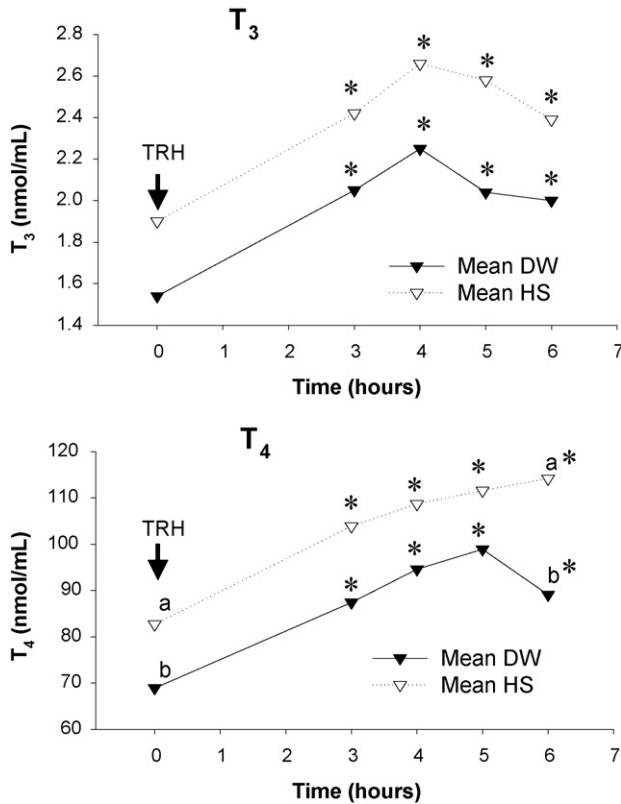
Fig. 6. Mean plasma concentrations of 3.5.3′-triiodothyronine (T_3) and thyroxine (T) of three dwarfs (DW) and four half-siblings (HS). Blood samples were collected between time 13:30 and 19:30. Immediately after the basal blood sample (0 h) was obtained (at time 13:30), thyroid-releasing hormone (TRH; 0.2 mg/mL) was intravenously administered (2 mL/animal) and further blood samples were obtained at 3, 4, 5 and 6 h after the injection. [*]Significant differences ($P < 0.05$) from basal levels at time 0. Different small letters indicate significant differences ($P < 0.05$) between DW and HS.

($P = 0.06$) in dwarfs than in half-siblings, and tended to be lower ($P = 0.08$) in half-siblings than in controls.

Across the replicated sampling periods and with two extractions per time period, reduced IGF-1R mRNA abundances were consistently observed in the dwarf group in samples from both liver as well as from muscle (Table 5). Abundance of hepatic mRNA for IGF1-R was 2.4-fold lower ($P = 0.003$) in dwarfs than in half-siblings, and 2.5-fold lower in dwarfs than in controls ($P = 0.001$). In skeletal muscle, IGF-1R mRNA abundance was 2.3-fold lower in dwarfs than in half-siblings ($P = 0.01$), and was 1.8-fold lower in dwarfs than in controls ($P = 0.08$).

The mRNA abundances of IGF-2 in liver tended to be lower ($P = 0.07$) in dwarfs than in controls and were numerically, but not significantly lower ($P = 0.11$) in half-siblings than in controls.
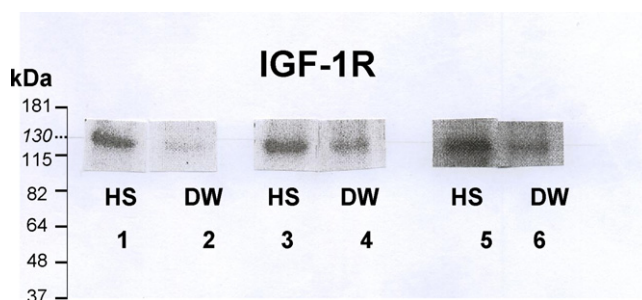
Fig. 7. Ligand (Western) blotting, performed using [^{125}I]-labelled insulin-like growth factor-1 (IGF-1) that was bound to the IGF type-1 receptor (IGF-1R), of liver tissue obtained by biopsies from three dwarf calves (DW) and three half-siblings (HS), performed in three different assays with one dwarf and one half-sibling per assay. Molecular weight markers were used to define the molecular weight of the IGF-1R (theoretically 130 kDa). Addition of non-radioactive IGF-1 (1 mg/mL) completely inhibited binding of [^{125}I]-IGF-1 to IGF-1R (not shown). Numbers and names of individual calves: 1 = Benni; 2 = Lulu; 3 = Björn; 4 = Poldi; 5 = Anna; 6 = Nepumuk (for additional details see legend to Fig. 1).

Levels of mRNA for GHR in muscle and of IR, IGF-1, IGF-2R, and IGFBP-1, -2, -3 and -4 in both liver and in muscle tissue were not significantly different among groups. The mRNA of IGFBP-5 and -6 were not detectable in either liver or muscle.

3.5. Insulin-like growth factor type-1 receptor at the protein level

When estimated by Western ligand blot technique, relative amounts of IGF-1R in the liver were 2.5 times higher ($P < 0.05$) in half-siblings than in dwarfs (ratios of band densities of Western ligand blots measured of three half-siblings (due to technical problems only three and not four Western ligand blots could be evaluated) relative to the dwarfs were 3.85, 1.93 and 1.71, respectively) (Fig. 7). The presence of IGF-1R in muscle was not cleanly detected in blots of homogenate preparations, suggesting that the abundance of this SA element was below the sensitivity of the method.

Immunostaining for IGF-1R in both the liver and muscle were largely structure-oriented and rather cell-specific (Fig. 8). In the liver, highest antigen (IGF-1R) intensities were evident in vascular and connective tissue, with lower pixel intensities evident throughout hepatocytes. Antigen intensity, estimated as color-specific pixels per field, were approximately five-fold higher in tissues from normal growing half-siblings compared with tissues from dwarfs ($P < 0.02$). Similarly, digital image analysis of muscle tissue further indicated that dwarfs presented antigenic IGF-1R in tissue structures at levels lower than those measured in half-siblings ($P < 0.07$). There were no obvious differences in pixel densities between males and females. Antigen presentation in muscle was lower in overall pixel intensity (muscle < 5% liver). As such, further image analysis for quantification purposes was performed with images captured with the 100× magnification rather than the 20× magnification as used with liver. The majority of IGF-1R antigen pixels in muscle were associated with vascular and connective tissues. The balance of specific immunostaining was associated with end regions and juxtaposed membranes between adjacent muscle fibers.
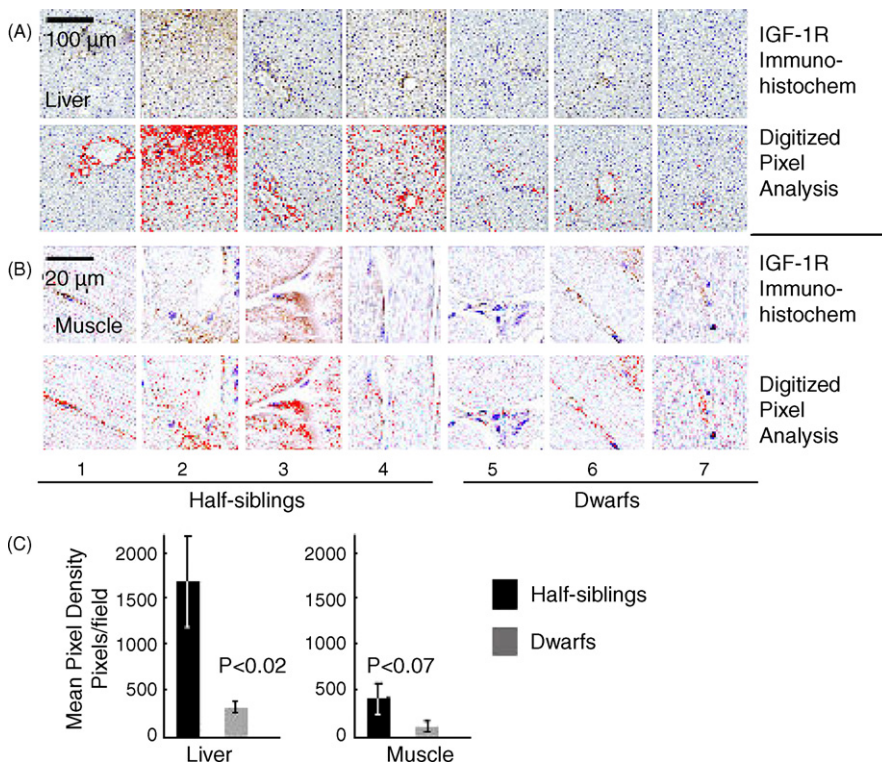
Fig. 8. Immunohistochemical analysis of IGF-Type 1 receptor (IGF-1R) in liver (section A) and anconeous muscle (section B) of calves (arbitrarily numbered 1 through 7) exhibiting normal and dwarf patterns of growth. For each set of tissue photographs (liver and muscle), the top panel represents actual DAB-based immunostaining (IGF-1R immunohistochemistry) while the bottom panel illustrates the digitized pixel analysis (vivid red) corresponding to the measured DAB staining. Summary statistics (means ± S.E.) derived from the quantification of pixel densities of each tissue type from half-siblings ($n = 4$) and dwarfs ($n = 3$) calves are presented in section C. Numbers and names of calves: 1 = Benni; 2 = Björn; 3 = Anna; 4 = Anita; 5 = Lulu; 6 = Poldi; 7 = Nepumuk (for additional details see legend to Fig. 1).

4. Discussion

While dwarfs clearly exhibited reduced body weight gain, importantly, they displayed proportional body growth as evidenced by the lack of differences in ratios of various body measurements compared with half-siblings and controls. There was no evidence for diseases that could have explained the reduced growth performance of dwarfs.

Although dwarf calves compared to half-siblings and control calves displayed slightly higher values for hemoglobin, hematocrit, total bilirubin, aspartate-amino-transferase and glutamate-dehydrogenase and plasma sodium concentrations were higher in dwarfs than in half-siblings, these changes were within clinico-chemical reference values [46,47] and thus could not be considered as grossly abnormal. Variation in some of these traits also gives evidence related to nutritional status. Because there were no gross deviations of dwarfs from

the normal ranges and from half-siblings and from controls, the nutritional status in dwarfs was adequate.

A dominant germline mutation as a cause of dwarfism seems likely as the dams of the dwarfs appeared not closely related and the normal service sire may be supposed to have transmitted the defective allele. An autosomal dominant inheritance can be excluded because all parents of the dwarfs showed normal growth and body weight for this breed. Arguments for a recessive inheritance would include a common ancestor or close relatedness among the sire and the dams of the affected calves, whereas, non-affected calves should not be inbred on this common ancestor. Segregation of dwarfism only in the progeny of this service sire corroborates the assumption of a germline mutation for this defect. Otherwise, in the case of Mendelian inheritance, dwarf calves would have been observed in previous cattle generations on this farm. Imprinting mechanism can be discussed as a further hypothesis for this pedigree and could have played a role. Clarification of the mode of inheritance needs further molecular genetic investigations. However, it was not possible to collect a semen sample of the service sire to prove the hypothesis of the germline mutation.

The most important finding of this study in our view was a reduced abundance of the IGF-1R gene expression in liver and skeletal muscle of dwarf calves and the reduced amount of hepatic IGF-1R at the protein level (based on Western blot analyses and immunohistochemical analyses). In immunohistochemical analyses the decreased intensity of antigen staining in dwarfs was not specific to any cell type as far as our ability to discriminate pixel densities allowed, but rather a generalized decrease in immunostaining in vascular, connective and other cell types. The low level of antigen in muscle, mainly visibly under the high power magnification, is consistent with the lack of signal for IGF-1R in muscle homogenates analyzed by Western ligand blot where the amount of reactive protein would be a fraction of the total protein extracted. Image analysis coupled with immunostaining provided the opportunity to evaluate the difference in IGF-1R in dwarf and half-sibling calves to a higher level of precision and structure-related association. A failure in the function of the somatotropic axis and especially a lack or a reduced expression and numbers of IGF-1R (followed by lacking or reduced post-receptor events) is expected to lead to reduced body weight gain, as was the case in the dwarf calves of this study.

It might be expected that reduced amounts of the IGF-1R would be accompanied by higher secretion of GH and IGF-1 due to interference with negative feedback mechanisms [48]. IGF-1R$^{+/-}$ knockout mice [49] and children possessing a mutation of IGF-1R [50] exhibit increased plasma GH and IGF-1 concentrations. Such a compensatory response could explain in part the higher mean GH concentrations and the greater integral GH responses to GRF-(1-30)-NH-ethyl acetate observed in the dwarf calves of this study. The IGF-1 concentrations among dwarfs, controls and half-siblings were, however, not significantly different. A failure of the negative feedback mechanism between GH and IGF-1 in dwarf calves was therefore not due to lowered expression of IGF-1R in the pituitary and hypothalamus.

The GH showed secretory episodes, as expected. Increased circulating plasma GH levels in dwarf calves of this study could be indicative of a defect in the GH receptor as shown in Laron syndrome in humans [3,4] or in cattle [28]. In those studies decreased circulating IGF-1 was observed, presumably due to a lack in the GHR. In the present case, however,

there were no significant differences of the IGF-1 status among the three groups and there was no evidence for a GHR defect.

In order to clarify the function of the somatropic axis, especially GH secretion, the α_2-adrenoceptor agonist xylazine was administered. Concentrations of GH increased and of insulin decreased, as expected [30,51,52]. Responses of GH and insulin to xylazine were not different among dwarfs, half-siblings, and controls. While plasma GH concentrations increased within 30 min as shown previously [30], IGF-1 levels in cattle did not increase until 5–6 h after the xylazine treatment. Similarly, in cows plasma GH concentrations increased after the administration of GRF, whereas, plasma IGF-1 concentrations remained stable [53]. Synthesis of IGF-1 after GH binding in the liver and sufficient release from hepatocytes to cause increased plasma concentrations appears to take hours, whereas GH is stored in the pituitary gland and released immediately.

The causes of the differences in plasma concentrations of glucagon and its consequences as well as its interpretations are uncertain at the moment. In cultured rat hepatocytes glucagon increases IGF-1 mRNA levels and IGF-1 secretion [54]. However published results are conflicting, and others report opposite findings, i.e. glucagon has also been demonstrated to inhibit IGF-1 production in rat hepatocytes [55].

The plasma concentrations of T_3 and T_4 were within physiological ranges [33,46,55]. The base-line plasma concentrations for T_4 were significantly lower in dwarfs than in half-siblings and likewise at the last sampling period after TRH administration. Concentrations of T_3 also tended to be lower in dwarfs than in half-siblings. Relationships of the thyroid hormones with the somatotropic axis are unclear. For example, in dogs chronic porcine GH treatment did not cause biologically significant changes in serum T_3 and T_4 levels [56]. Likewise, transient decreases in T_4 as well as unchanged T_3 concentrations were observed after treatment of humans with recombinant human GH [57]. Nevertheless, GH treatment has also been shown to enhance the peripheral conversion of T_4 to T_3 in children [58]. Studies in normal cattle showed no relationship between thyroid hormones and plasma GH, IGF-1 plasma concentration, or liver IGF-1 mRNA [59]. The decreases of T_3 and T_4 concentrations in the present study were small and within the physiological range and were unlikely to be responsible for changes of the somatotropic axis and for reduced growth performance of these dwarf calves.

In order to elucidate the genetic cause for proportional growth retardation in dwarf calves, mRNA expression of genes of the somatotropic axis other than of IGF-1R was investigated. A significant decrease in mRNA abundance for GHR was found in dwarfs in the liver, but not in skeletal muscle. These results are basically very similar to those observed in studies of Efe pygmies in which the growth defect was determined to be caused by reduced expression of IGF-1R as the primary defect, along with GH resistance and accompanied by insulin resistance [6].

In conclusion, in three dwarf calves without any obvious further clinical defect, decreased IGF-1R amounts at the mRNA and protein level were identified in both liver and skeletal muscle tissue. The GHR expression was also down-regulated, but significantly only in liver, possibly in part as a consequence of the slightly enhanced plasma GH status. The contribution of additional endocrine changes (insulin, glucagon, T_4, T_3), which were in the physiological ranges, to induce dwarfism in this study is less clear, but probably not of primary importance. At present, decreased mRNA and protein levels of IGF-1R are the

most likely cause that could explain short stature and proportional growth retardation in dwarf calves in this study. The aetiology thus appears to be different from those described as likely causes of bovine dwarfism in other studies. Further work is required in order to provide additional evidence for this IGF-1R defect. It will be particularly useful to sequence the IGF-1R gene to identify possible mutations that cause this type of dwarfism.

Acknowledgments

References

[1] Schneider HJ, Pagotto U, Stalla GK. Central effects of the somatotropic system. Eur J Endocrinol 2003;149:377–92.

[2] Besson A, Salemi S, Gallati S, Jenal A, Horn R, Mullis PS, Mullis PE. Reduced longevity in untreated patients with isolated growth hormone deficiency. J Clin Endocrinol Metab 2003;88:3664–7.

[3] Laron Z, Pertzelan A, Mannheimer S. Genetic pituitary dwarfism with high serum concentration of growth hormone—a new inborn error of metabolism? Isr J Med Sci 1966;2:152–5.

[4] Laron Z. Insulin-like growth factor 1 (IGF-1): a growth hormone. Mol Pathol 2001;54:311–6.

[5] Peoples R, Milatovich A, Francke U. Hemizygosity at the insulin-like growth factor I receptor (IGF1R) locus and growth failure in the ring chromosome 15 syndrome. Cytogenet Cell Genet 1995;70:228–34.

[6] Hattori Y, Vera JC, Rivas CI, Bersch N, Bailey RC, Geffner ME, Golde DW. Decreased insulin-like growth factor I receptor expression and function in immortalized African Pygmy T-cells. J Clin Endocrinol Metab 1996;81:2257–63.

[7] Binder G, Mavridou K, Wollmann HA, Eggermann T, Ranke MB. Screening for insulin-like growth factor-I receptor mutations in patients with Silver-Russell syndrome. J Pediatr Endocrinol Metab 2002;15:1167–71.

[8] Abuzzahab MJ, Schneider A, Goddard A, Grigorescu F, Lautier C, Keller E, Kiess W, Klammt J, Kartzsch J, Osgood D, Pfaffle R, Raile K, Seidel B, Smith RJ, Chernausek SD. IGF-I receptor mutations resulting in intrauterine and postnatal growth retardation. N Engl J Med 2003;349(4):2211–22.

[9] Andresen E, Willeberg P. Pituitary dwarfism in German shepherd dogs: additional evidence of simple, autosomal recessive inheritance. Nord Vet Med 1976;28:481–6.

[10] Japp RG, Mohammadian M. Sex-linked dwarfism and egg production of broiler dams. Poult Sci 1969;48:344–6.

[11] Jensen PT, Nielsen DH, Jensen P, Bille N. Hereditary dwarfism in pigs. Nord Vet Med 1984;36:32–7.

[12] Séllier P. Genetically caused retarded growth in animals. Domest Anim Endocrinol 2000;19:105–19.

[13] Mead SW, Gregory PW, Regan WM. Proportionate dwarfism in Jersey cows. J Hered 1942;33:411–6.

[14] Johnson LE, Harshfield GS, McCone W. Dwarfism, a hereditary defect in beef cattle. J Hered 1950;41:177–81.

[15] Lörtscher H. Das Auftreten von Zwergwuchs und dessen Bekämpfung beim Simmentaler Fleckvieh. Schweiz Fleckviehzuchtverband 1957;5:23–4.

[16] Witt M, Schilling E, Huth F-W. Zwergwuchs bei schwarzbunten Rindern. Z Tierzüchtg Züchtbiol 1959;73:201–7.

[17] Gottwald W. Über das Vorkommen von Zwergwuchs in der Nachzucht eines Fleckviehbullen. Zuchthyg 1967;2:63–7.

[18] Gregory KE, Spahr SL. Miniature calves. J Hered 1979;70:217–9.

[19] Pirchner F, Kaiser E. Proportionierter Zwergwuchs bei Fleckvieh. Wien tierärztl Wschr 1986;73:173–7.

[20] Distl O, Schams D, Graf F, Meyer J, Kräusslich H. Untersuchungen an Kälbern mit proportioniertem Zwergwuchs. Tierärztl Umschau 1990;45:727–8.

[21] Hammond AC, Elsasser TH, Olson TA. Endocrine characteristics of a miniature condition in Brahman cattle: circulating concentrations of some growth-related hormones. Proc Soc Exp Biol Med 1991;197:450–7.

[22] Harper PA, Latter MR. Proportionate dwarfism in Angus cattle. Aust Vet J 1993;70:450.

[23] Deinhofer M, Majemnik C. Insulin-like growth factor I and insulin-like growth factor binding protein-3 in a steer with growth retardation. Dtsch Tierärztl Wschr 1996;103:130–1.

[24] Usha AP, Lester DH, Williams JL. Dwarfism in Dexter cattle is not caused by the mutations in FGFR3 responsible for achondroplasia in humans. Anim Genet 1997;28:55–7.

[25] Blum JW, Kovacs B, Nett P, Schenk M, Stranzinger G. Metabolic and endocrine changes in a dwarf calf. In: Blum JW, Elsasser T, Guilloteau P, editors. Proceedings of the symposium on growth in ruminants: basic aspects, theory and practice for the future. Switzerland: University of Bern; 1998. p. 26 (Abstract).

[26] Hammond AC, Elsasser TH, Lucy MC, Chase Jr CC, Olson TA. Dwarf and miniature models in cattle. In: Blum JW, Elsasser T, Guilloteau P, editors. Proceedings of the symposium on growth in ruminants: basic aspects, theory and practice for the future. Switzerland: University of Bern; 1998. p. 188–96.

[27] Harper PA, Latter MR, Nicholas FW, Cook RW, Gill PA. Chondrodysplasia in Australian Dexter cattle. Aust Vet J 1998;76:199–202.

[28] Liu J, Boyd CK, Kobayashi Y, Chase Jr CC, Hammond AC, Olson TA, Elsasser TH, Lucy MC. A novel phenotype for Laron dwarfism in miniature *Bos indicus* cattle suggests that the expression of growth hormone receptor 1A in liver is required for normal growth. Domest Anim Endocrinol 1999;17:421–37.

[29] Kitagawa H, Kitoh K, Ito T, Ohba Y, Nishii N, Katoh K, Obara Y, Motoi Y, Sasaki Y. Serum growth hormone and insulin-like growth factor-1 concentrations in Japanese black cattle with growth retardation. J Vet Med Sci 2001;63:167–70.

[30] Scholtysik G, Regli F, Bruckmaier RM, Blum JW. The α_2-adrenoceptor agonists xylazine and guanfacine exert different central nervous system, but comparable peripheral effects in calves. J Vet Pharmacol Therap 1998;21:477–84.

[31] Hammon HM, Blum JW. The somatotropic axis in neonatal calves can be modulated by nutrition, growth hormone, and Long-R^3-IGF-I. Am J Physiol 1997;273:E130–8.

[32] Hammon HM, Blum JW. Metabolic and endocrine traits of neonatal calves are influenced by feeding colostrum for different durations or only milk replacer. J Nutr 1998;128:624–32.

[33] Rérat M, Zbinden Y, Saner R, Hammon H, Blum JW. In vitro embryo production: growth performance, feed efficiency, and hematological, metabolic and endocrine status in calves. J Dairy Sci 2005;88:2579–93.

[34] Rauprich ABE, Hammon HM, Blum JW. Effects of feeding colostrum and a formula with nutrient contents as colostrum on metabolic and endocrine traits in neonatal calves. Biol Neonate 2000;78:53–64.

[35] Mudron P. Vitamin-E-Status bei Kühen mit linksseitiger Labmagenverlagerung und Fettleber. Thesis Med Vet, Tierärztliche Hochschule Hannover, 1994.

[36] Witte B. Untersuchungen zum Gehalt von Thiamin und seinen Derivaten in Vollblut und Blutplasma bei Rindern sowie deren Verhältnis zum Transketolasetest. Thesis Med Vet, Tierärztliche Hochschule Hannover, 1998.

[37] Cribiu EP, Di Berardino D, Di Meo GP, Eggen A, Gallagher DS, Gustavsson I, Hayes H, Iannuzzi L, Popescu CP, Rubes J, Schmutz S, Stranzinger G, Vaiman A, Womack J. International system for chromosome nomenclature of domestic bovids (ISCNDB 2000). Cytogenet Cell Genet 2001;92:283–99.

[38] Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. Anal Biochem 1987;162:156–9.

[39] Pfaffl MW, Georgieva TM, Georgiev IP, Ontsouka E, Hageleit M, Blum JW. Real-time RT-PCR quantification of insulin-like growth factor (IGF)-1, IGF-1 receptor, IGF-2, IGF-2 receptor, insulin receptor, growth hormone receptor, IGF-binding proteins 1–3 in the bovine species. Domest Anim Endocrinol 2002;22:91–102.

[40] Plath-Gabler A, Gabler C, Sinowatz F, Berisha B, Schams D. The expression of the IGF family and GH receptor in the bovine mammary gland. J Endocrinol 2001;168:39–48.

[41] Hammon HM, Sauter SN, Reist M, Zbinden Y, Philipona C, Morel C, Blum JW. Dexamethasone and colostrum feeding affect hepatic gluconeogenic enzymes differently in neonatal calves. J Anim Sci 2003;81:3095–106.

[42] Hammon HM, Blum JW. Feeding different amounts of colostrum or only milk replacer modify receptors of intestinal insulin-like growth factors and insulin in neonatal calves. Domest Anim Endocrinol 2002;22:155–68.

[43] Elsasser TH, Kahl S, MacLeod C, Nicholson B, Sartin JL, Li C. Mechanisms underlying growth hormone effects in augmenting nitric oxide production and protein tyrosine nitration during endotoxin challenge. Endocrinology 2004;145:3413–23.

[44] SAS. Introductory comparison of the GLM and mixed procedure. Cary, NC, USA: SAS Institute Inc.; 1994.

[45] Merriam GR, Wachter KW. Algorithms for the study of episodic hormone secretion. Am J Physiol 1982;243:E310–8.

[46] Kraft W, Dürr UM. Klinische Labordiagnostik in der Tiermedizin. Schattauer–Verlag; 1997.

[47] Dirksen G, Gründer H-D, Stöber M. Innere Medizin und Chirurgie des Rindes. 4th ed. Berlin and Vienna: Paul Parey/Blackwell Publishing; 2002.

[48] Giustina A, Veldhuis JD. Pathophysiology of the neuroregulation of growth hormone secretion in experimental animals and the human. Endocr Rev 1998;19:717–97.

[49] Holzenberger M, Dupont J, Ducos B, Leneuve P, Geloen A, Even PC, Cervera P, Le Bouc Y. IGF-1 receptor regulates lifespan and resistance to oxidative stress in mice. Nature 2003;421:182–7.

[50] Bierich JR, Moeller H, Ranke MB, Rosenfeld RG. Pseudopituitary dwarfism due to resistance to somatomedin: a new syndrome. Eur J Pediatr 1984;142:186–8.

[51] Kasuya E, Hodate K, Matsumoto M, Sakaguchi M, Hashizume T, Kanematsu S. The effects of xylazine on plasma concentrations of growth hormone, insulin-like growth factor-I, glucose and insulin in calves. Endocr J 1996;43:145–9.

[52] Hodate K, Johke T, Ozawa A, Ohashi S. Plasma growth hormone, insulin-like growth factor-I, and milk production responses to exogenous human growth hormone-releasing factor analogs in dairy cows. Endocrinol Jpn 1990;37:261–73.

[53] Kachra Z, Barash I, Yannopoulos C, Khan MN, Guyda HJ, Posner BI. The differential regulation by glucagon and growth hormone of insulin-like growth factor (IGF)-I and IGF binding proteins in cultured rat hepatocytes. Endocrinology 1991;128:1723–30.

[54] Denver RJ, Nicoll CS. Pancreatic hormones differentially regulate insulin-like growth factor (IGF)-I and IGF-binding protein production by primary rat hepatocytes. J Endocrinol 1994;142:299–310.

[55] Blum JW, Schnyder W, Kunz PL, Blom AK, Bickel H, Schürch A. Reduced and compensatory growth: endocrine and metabolic changes during energy restriction and realimentation in steers. J Nutr 1985;115:417–25.

[56] Prahalada S, Stabinski LG, Chen HY, Morrissey RE, De Burlet G, Holder D, Patrick DH, Peter CP, van Zwieten MJ. Pharmacological and toxicological effects of chronic porcine growth hormone administration in dogs. Toxicol Pathol 1998;26:185–200.

[57] Kalina-Faska B, Kalina M, Koehler B. Effects of recombinant growth hormone therapy on thyroid hormone concentrations. Int J Clin Pharmacol Ther 2004;42:30–4.

[58] Pirazzoli P, Cacciari E, Mandini M, Sganga T, Capelli M, Cicognani A, Gualandi S. Growth and thyroid function in children treated with growth hormone. J Pediatr 1992;121:210–3.

[59] Hannon K, Trenkle A. Relationship of thyroid status to growth hormone and insulin-like growth factor-I (IGF-I) in plasma and IGF-I mRNA in liver and skeletal muscle of cattle. Domest Anim Endocrinol 1991;8:595–600.